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Optimization of Polyhydroxybutyrate (PHB) Production by Locally Isolated *Bacillus aryabhattai* Using Response Surface Methodology

Neveen M. El-Metwally[1*], Reda F. Allam[1], Mona S. Shafei[1], Magdy A. Amin[2], Yasser M. Ragab[2], Heba A. El-Refai[1] and Abd El-Nasser A. Khattab[3]

[1]Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Giza 12622, Egypt.
[2]Department of Microbiology and Immunology, Cairo University.
[3]Genetics and Cytology Department, National Research Centre, Dokki, Giza 12622, Egypt.
*Corresponding author: neveen_elmetwally@yahoo.com

Abstract

In the present study, an attempt was made to isolate an efficient Polyhydroxybutyrate producing bacterium from soil. A total of 38 different types of bacteria were isolated, out of which 15 were found to be PHB positive, based on the viable colony staining method of screening using Nile red Dye. The isolate (6N) showed maximum PHB production of 0.17 g/L, and PHB produced was confirmedusing NMR.The most potent isolate (6N-NRC) was identified using 16S rRNA, and phylogenetic analysis clearly demonstrated that the strain 6N-NRC is a member of the genus *Bacillus* and is identified as *Bacillus aryabhattai*. The culture medium and growth parameters were optimized using one factor at a time, multifactorial experimental design (Plackett-Burman and Box-Behnken) and utilization of Beet molasses as cheap and economic carbon source for maximum PHB production was done. Beet molasses (30 g/L) as the carbon and ammonium chloride (0.75 g/L) as the nitrogen source were found to be the best nutritional sources for maximum PHB production. Incubation time period 36h, pH of the medium at 8.0 and temperature of 30^0C were found to be optimum conditions for obtaining maximum PHB yield of 3.799 g/L.

Key words : Polyhydroxybutyrate (PHB), NMR, *Bacillus aryabhattai*, multifactorial experimental design, Beet molasses.

Introduction

Plastics like polypropylene, polyethylene and polystyrene are almost made from petroleum which is the main source of energy (1). Around 270 million metric tons per year of fossil fuels are consumed for manufacturing plastics (2). The consumption of petroleum at this rate will lead to its depletion in the next 60-80 years (3). On the other hand, the environmental accumulation of plastics has become a worldwide problem (4).

Consequently, petroleum based plastics were replaced by biodegradable polymers which are considered ecologically as useful alternatives to plastics (5). Poly-3-hydroxy butyric acid (PHB)is the common type of polyhydroxy alkonoates (PHA) from which bioplastics are made (6). PHB has unique properties such as UV resistant, insoluble in water, oxygen permeability and highly resistant to hydrolytic degradation, poorly resistant to acids and bases, soluble in chloroform and other chlorinated hydrocarbons and thus it is used in medical applications (7). PHBs are considered as to be sources for

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biodegradable and biocompatible plastic materials (8). Thus, the current problems causedby decreasing the nonrenewable energy resources and environmental pollution caused by plastic garbage are reduced. PHB are also used in material science, food industries and agriculture (9).

A large number of microorganisms have the ability to synthesize PHB as intracellular energy reserve material under certain limitations of some essential nutrients such as nitrogen, magnesium and phosphate in presence of excess carbon sources (10). Many kinds of microbes such as *Bacillus* sp. (11), *Pseudomonas* (12), *Azotobacter- vinelandii* (13), *Sinorhizobium meliloti* and *Escherichia coli* (13, 14) are able to generate PHB.

The production of PHB either on the industrial or commercial scale is limited due to the relatively high cost of the utilized substrate compared to synthetic plastic (9).Hence, the strategy of biodegradable plastic production is important for PHB production as it relies on isolating most efficient PHB producing bacterial strain and optimizing the cultural parameters (5).

The purpose of this work was to isolate and identify a local bacterial strain capable of producing PHB followed by identification of the best isolate by 16s rRNA sequence analysis and phenotypic characterization. The one-factor-at-a time experiments were done to choose the optimum carbon and nitrogen sources. Then, Plackett-Burman design was used to identify the most significant variables affecting production of PHB. Moreover, Box-Behnken was done to optimize the effective variables. Furthermore, the chemical structure of PHB synthesized by the most potent isolate was determined by NMR spectroscopy analysis.

Materials and Methods

Sample collection and isolation of pure cultures: Microbial isolates were recovered from six different soil samples originating from different Egyptian cities and given the symbols as follows: El Menoufia (A), El Wadi El Gedid (E), El Fayoum (G)and El Giza (J, K and N). One gram of soil sample is dispensedin 10mls of sterile distilled water, mixed vigorously and 1ml from this is taken and added to another tube with 9mls sterile distilled water to get a dilution of 10^{-1}. This serial dilution is repeated to get dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6}$and 10^{-7}. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient agar medium by spread plate method for the propagation of microbial growth. The plates were incubated at 30°C for 48 hours. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4°C (15).

Screening of PHB producing isolates by nile red dye: All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Nile red Dye. For this screening of PHB producers, 20 µl was spread onto sterilized pre-made (minimal salt agar media) plates to reach a final concentrations of 0.5 µg Nile red /ml medium. After inoculation, the plates were incubated overnight at 30 ºC subsequently. The prepared clay soil samples were subcultured by 0.1 ml samples and then spread out with a sterilized glass rod over the surface of minimal salt agar media. The plates were incubated at 30 ºC for 48h. Colonies with pinkish pigment indicated PHB production isolates were exposed to ultraviolet light (312 nm) to detect the accumulation of PHB according to the lighted plates and were given positive signs. After that these isolates were picked up and purified by sub-cultured on the same media (16).

Determination of cell dry weight: After 48h incubation at 30°C, culture medium was collected and the cell dry weight was measured by centrifugationof 100ml of the culture at 10,000 rpm for 15min at 4ºC. Supernatant was discarded and the cell pellets werewashed twice in deionized water and dried at 80ºC until a constant weight then the total bacterial cell dry weight was determined as g/l (17).

Production medium: Minimal Salt Media (MSM) (18) was prepared as follows:(KH_2PO_4 1.5g; Na_2HPO_4 3.525g; $MgSO_4.7H_2O$ 0.2g; $CaCl_2$0.02g; ferric citrate 0.0015g; glucose 20g; NH_4Cl 0.75g; trace elements solution 1ml; distilled water 1000 ml; pH 7.5) for the production of poly- β–hydroxybutyrate. MSM (100 ml) was taken in each Erlenmeyer flask and autoclaved at 121°C for 20 min after which 10% (v/v) of fresh bacterial inoculum were inoculated in each flask and incubated for 7 days at 30°C.

Extraction and quantification of PHB: Polyhydroxybutyrate polymer was extracted and the amount of PHB produced was calculated from the standard curve prepared by using commercial poly-β-hydroxybutyrate (Sigma-Aldrich) as per the method detailed by Law and Slepecky (19). All the PHB positive bacterial isolates were inoculated in minimal salt medium and the cell growth of each isolate containing the polymer was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet was washed with acetone and ethanol to remove the unwanted materials, resuspended in equal volume of 4 % sodium hypochlorite and incubated at 37°C for 24h. The mixture was then centrifuged at 10,000 rpm for 10 min to sediment the lipid granules. The supernatant was discarded, and the cell pellet was washed successively with acetone, ethanol and water to remove unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally, the pelleted polymer granules were dissolved in hot chloroform and filtered through Whatmann no. 1 filter paper (previously treated with hot chloroform). To the filtrate, 10 ml of hot concentrated H_2SO_4 were added, which converts the polymer to crotonic acid, turning it into a brown colored solution. The solution was cooled and absorbance was read at 235nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer. The quantity of PHB produced was determined by referring to the standard curve (5).

Molecular identification of selected PHB producing bacterial isolate: The genomic deoxyribonucleic acid (RNA) was extracted from isolated culture of *Bacillus* strain (6N-NRC) by using the protocol of Gene JET Genomic DNA Purification Kit (Thermo K0721,Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). The PCR amplification of 16S ribosomal gene was performed by using Maxima Hot Start PCR Master Mix (Thermo K1051) and the nucleotide sequences of the 16S primersused are: forward primer-5'-AGAGTTTGATCCTGGCTCAG-3'and reverse primer-5' GGTTACCTTGTTACGACTT -3' (20). To each PCR vial containing 10 µL of 2X PCR Master Mix, 2 µL of each used primer (10 pmole/µl) and 2 µL of the purified DNA sample (40 ng/ µl) were added. The total volume of the amplification reaction was completed to 20 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95°C for five min. Thirty-five cycles each consists of the following segments: Denaturation at 95°C for one min; primer annealing for two min. at 52°C and polymerization at 72°C for two min. Finally, hold the PCR at 4°C. The PCR product was cleaned up using Gene JET™ PCR Purification Kit (Thermo K0701). The DNA sequencing of the PCR product was carried out by using Applied Biosystems (ABI) 3730xl DNA sequencer (GATC Biotech, Constance Germany) by using forward and reverse primers.

Phylogenetic analysis : The 16S ribosomal DNA (rRNA) sequences of the strain (6N-NRC) compared with the sequences available in National Center for Biotechnology Information (NCBI), Gene Bank database by using the Basic Local Alignment Search Tool (BLAST). The sequences were aligned together with those of reference taxa retrieved from public databases. The evolutionary distances were set up based on parameter model and phylogenetic tree was constructed by using the neighbor-joining method MEGA6 software (21, 22). The 16S rRNA sequence was submitted to the NCBI Gene Bankwith nucleotide sequence database under accession number MH997667.1

Nuclear magnetic resonance (NMR) analysis: The proton Nuclear Magnetic Resonance

Spectroscopy (^{1}H NMR) of the polymer was recorded after suspending the polymer in high purity deuterochloroform ($CDCl_3$). 1H NMR spectra were obtained in model Bruker High Performance Digital FT-NMR spectrometer AvanceIII 400 MHz at 20-25°C, 4.0894465s acquisition time and 8012.820 Hz spectral width.

Optimization of cultural parameters for maximum PHB production: Different factors affecting PHB production by the selected bacterial isolate were optimized using one factor at a time and multi-factorial design techniques.

Optimization using one factor at a time (OFAT)

Effect of different incubation periods: The bacterial isolate was grown in flask (250 ml) with 100 ml minimal salt medium at pH 7.0 and was sterilized at 121°C for 20 min The inoculated flasks with 10% v/v were incubated at 30°C at 200 rpm under different incubation periods (1,2,3,4, 5,6,7, 8,9 and 10 days), PHB produced was quantified.

Effect of different carbon sources : The effect of different carbon sources on PHB production was determined by inoculating the bacterial isolate in 100 ml of minimal salt medium (MSM) (18) supplemented with different carbon sources such as glucose, fructose, sucrose, maltose, arabinose, galactose and gluconic acid at 2% concentration. Cheap carbon sources like sugarcane and beet molasses were also tested as carbon sources. Cultures were incubated at 30°C on a rotary shaker (200 rpm) for 48 h. After incubation, PHBs produced were quantified.

Effect of different nitrogen sources: The bacterial isolate was grown in 100 ml of MSM broth containing the optimum carbon source and different organic and inorganic nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate, urea, casein, yeast, corn steep liquor, beef extract and peptone) were used at nitrogen base concentration. After 48 h of incubation at 30°C, PHB yield was determined, and the optimum nitrogen source was selected.

Effect of different inoculum size: Different inoculum sizes (1,2,3,4,5,6,7,8,9 and 15 %v/v) of bacterial isolate were grown in 100 ml of MSM broth. After 48 h of incubation at 30°C, PHB yield was determined, and the optimum inoculum size was selected.

Statistics: All experiments were performed in triplicates, the data shown in the corresponding tables and figures were the mean values of the experiments and the relative standard deviations were shown (mean ± SE).

Multifactorial experimental design and optimization: Experimental design as two steps sequential optimization is used to screen many variables together in one experiment and to optimize them for a desired response in a much faster way than examining one variable at a time.

Plackett-Burman design : Plackett-Burman Design (PBD) was employed for selection of significant variables in PHB production. Application of statistical methods involving Plackett-Burman Design (PBD) has gained a lot of impetus for medium optimization (23). Plackett-Burman design was used to screen the most significant parameters affecting PHB production. This design is recommend when more than five factors are under investigation (24). Seven independent variables were screened in nine combinations, organized according to the Plackett-Burman design matrix. For each variable, a high level (+) and low level (-) was tested. All trials were performed in triplets and their averages were treated as the responses.The main effect of each variable was determined by the following equation:

$$E_{xi} = (\ _{Mi+} - \ _{Mi-}) / N$$

Where E_{xi} is the variable main effect, Mi+ and Mi- are either PHB production in g/l or dry cell weight of the selected isolate or PHB yield % in trials where the independent variable (xi) was present in high and in low settings, respectively, and N is the number of trials divided by 2.

Box-Behnken design: In the second phase of medium formulation for maximum PHB production,

the Box-Behnken experimental design was applied where the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). Thirteen combinations and their observations were fitted to the following second order polynomial mode:

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where, Y is the dependent variable (PHB production); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at centerpoint; b_1, $b_2$and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients.The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R^2 (regression coefficient). If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum operational conditions (25, 26). The 3D graphs were generated to understand the effect of selected variables individually and incombination to determine their optimum level for maximal PHB production. The F-test was performed to determine factors having a significant effect ($P< 0.1$).

Results and Discussion

Isolation and screening of PHB producing microorganisms: In this study microorganisms were isolated from clay soilsamples using serial dilution. Atotal of 38 bacterial colonies with different morphological features were selectedand given numbers.Bacterial colonies were preserved on enriched nutrient agar medium to be studied (Table 1). Among 38 colonies, 15 colonies showed positive pinkish colony for Nile red staining. The bacterial strains were further evaluated for PHB production by preliminary screening using submerged fermentation technique; quantification was done spectrophotometrically and by comparing the absorbance readings with a standard crotonic acid curve. The cell dry weight (g/L) and PHB yield % were studied for the 15 positive isolates (Table 2). The colonies of the most potent isolate (6N) grown on minimal salt medium containing Nile red dye under ultraviolet light (UV) showed pink fluorescence which indicated the presence of PHB. The PHB yield % ranged from 12.8-38.63% and the highest percentage was observed by the isolate designated as 6N. Bacteria belonging to *Bacillus* were known previously to accumulate high concentrations of PHA (27, 28). Similar results were obtained by Aly*et al.* (6) who studied the production of PHB using *B. cereus* MM7 isolated from soil samples. Higher results were shown by Bhuwal *et al.* (29) who estimated the maximum PHA production was 79.27% and 77.63% using *Enterococcus* sp. and *Brevundimonas* sp. respectively isolated from cardboard industry waste water. In literature, both gram positive and gram negative bacteria are capable to accumulate PHB (30). Similar results show that the natural environment has been an extensive area of research for the production of PHB (31). Different environments including soil (32), sea water and deep sea mud (33)and oil sludge (34) represent rich areas for extensive screening and identification of gram positive and gram negative bacteria for PHB production.

Molecular characterization of the selected isolate: 16S rRNA gene investigation was performed. The 16S forward and reverse primers were used to amplify the region of the 16S ribosomal ribonucleic acid (rDNA) gene from the genomic DNA of the *Bacillus* (6N-NRC). After the amplification by PCR, a product of nearly 1500 bp was obtained. The BLAST analysis of the amplified 16S rDNA gene sequence revealed 99% similarity to the partial 16S rDNA gene of *Bacillus aryabhattai* B8W22 strain. Phylogenetic analysis clearly demonstrated that strain 6N-NRC is a member of the genus *Bacillus* and is identified as *Bacillus aryabhattai* (Fig. 1). It is known that bacteria belonging to *Bacillus* produce a high PHA

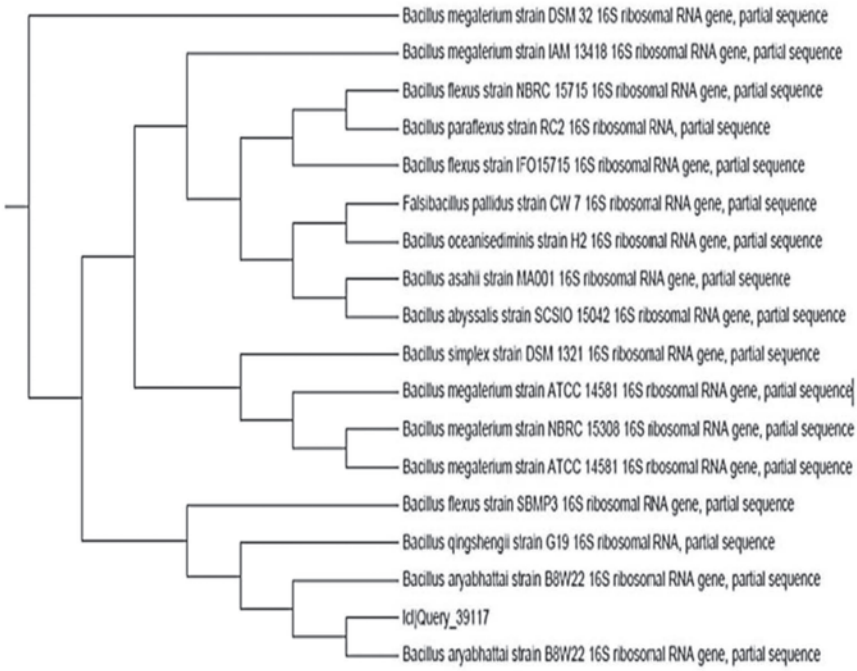

Fig. 1. Phylogenetic tree of the efficient *Bacillus* strain (6N-NRC) producing PHB in comparison to the most related bacterial strain *(Bacillus aryabhattai)* in database.

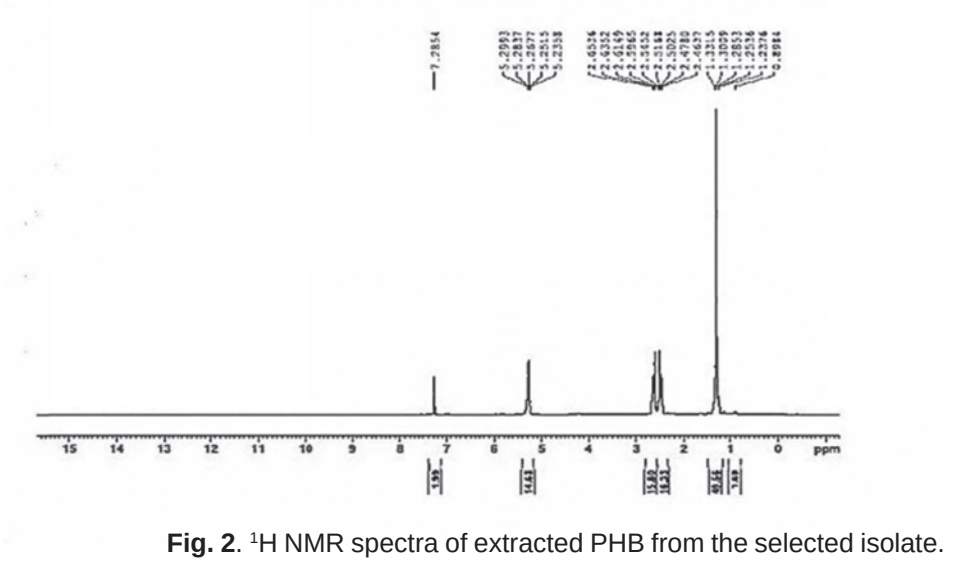

Fig. 2. ^{1}H NMR spectra of extracted PHB from the selected isolate.

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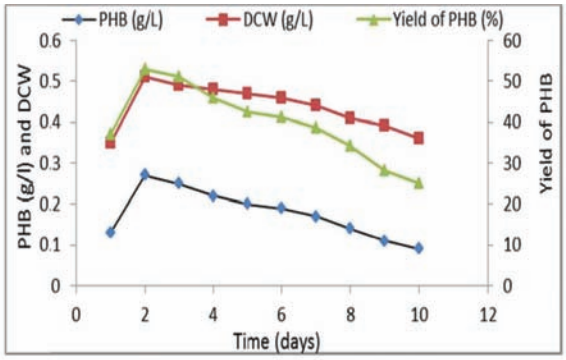

Fig. 3. Effect of incubation period on production of PHB using *Bacillus aryabhattai* 6N-NRC

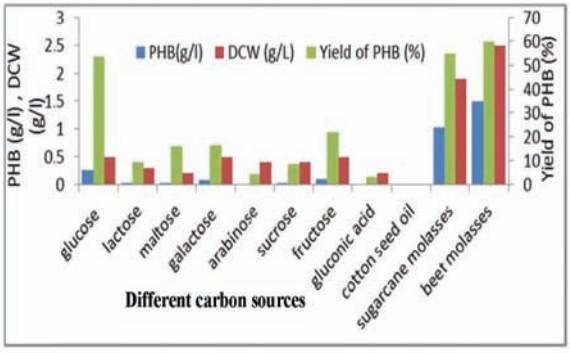

Fig. 4. Effect of different sources of carbon on production of PHB by *Bacillus aryabhattai* 6N-NRC

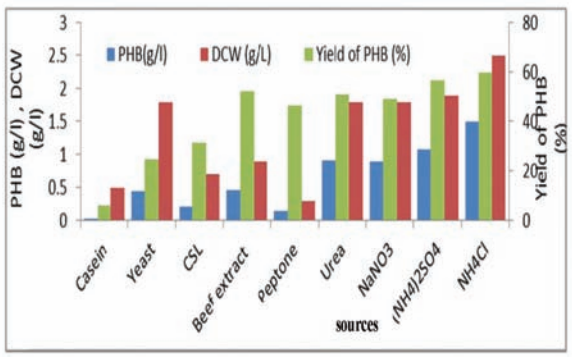

Fig. 5. Effect of different sources of nitrogen on production of PHB by *Bacillus aryabhattai* 6N-NRC

concentration [27, 28]. *Bacillus* sp. has the ability to synthesize both short and medium chain length PHAs (35).

Polymer analysis by ^{1}H-NMR spectroscopy : Based on NMR characterization of the PHA produced by selected bacterial isolate in comparison with the standard PHB (Sigma),it was found that the properties PHA produced are similar to that of the standard PHB(Sigma) (Fig. 2), so the PHA produced is polyhydroxybutyrate (PHB). The structure of polyester was investigated by ^{1}H NMR. The ^{1}H NMR spectra of the PHA extracted from selected strain show thefollowing resonance signals: HC=CH bond at 5.26 ppm ,CH_2O–COOH bond at 2.56 ppm, a high signal at 1.28 ppm that belongs to the hydrogen of methylene in the saturatedlateral chain, and a terminal –CH_3 group at 0.89 ppm (36).Three groups of signals characterizing PHB: a douplet at 1.28 ppm which is attributed to the methyl group, a doublet of quartet at 2.56 ppm which is attributed to methylene group and a multiplet at 5.26 ppm, which is characteristic of methine group. The ^{1}HNMR spectra of the sample and the standard are almost identical, which confirms that produced compound is polyhydroxybutyrate (PHB).

Optimization of culture medium for maximum PHB production: The nutritional, growth and physical factors such as the C-source, N-source, incubation time, pH and temperature greatly affect PHB accumulation (37). Therefore, these parameters were examined for maximum PHB production by the selected isolate.

Effect of incubation periods: The effect of time of incubation on production of PHBby the selected isolate was shown in Fig. 3. The optimum incubation period for the selected isolate was 48h where the PHB yield was 52.94% (w/w). This agrees with Hawas*et al*. (15), Valappil*et al*. (38), Kumar *et al*. (39) and Berekaa and Al Thawadi (40). On contrary,Pillai*et al*. [41] reported that *Bacillus aryabhattai*reached maximum polymer accumulation after 60 h. Other researchers found

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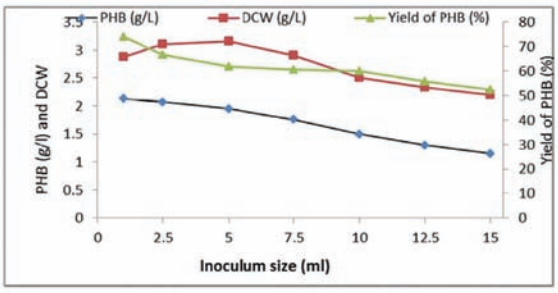

Fig. 6. Effect of inoculum size on production of PHB by *Bacillus aryabhattai* 6N-NRC

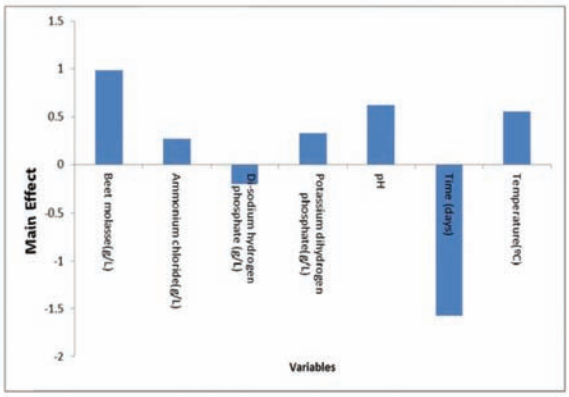

Fig. 7. Main effects of variables on PHB production by Bacillus aryabhattai

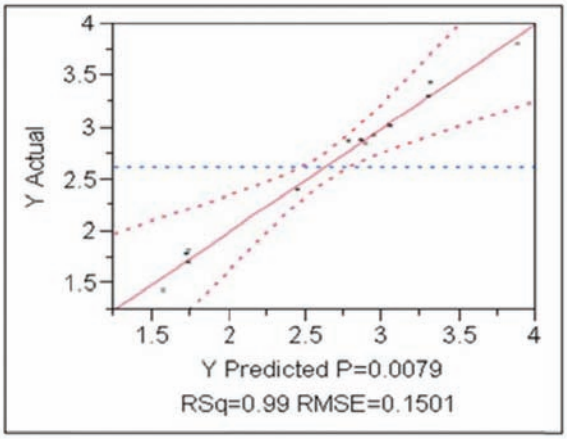

Fig. 8. Response Actual by Predicted plot of PHB production

that 24h was the best incubation period (42, 43, 44).

Previously, it was reported that bacteria which produce PHB were divided into two groups. The first groupincludes those which produce PHB during stationary phase when oxygen, magnesium, phosphorous and nitrogen are limited while the carbon source is present in great amounts. The second group involves PHB production in the growth phase (45). The Bacillus cereus belongs to the first group. In this study, the fermentation was for different incubation periods from 1 to 10 days. After 48h, maximum PHB accumulation was shown. This may be attributed to the acclimatization phase. Cell mass increased steadily, leading to maximum production at 48h followed by gradual decrease. This was due to presence of bacteria in its decline stage due to the decrease of nutrient supplements and accumulation of metabolites, toxins and inhibitors (38).

Effect of different sources of carbon : The effect of different sources of carbon (glucose, lactose, maltose, galactose, arabinose, sucrose, fructose, gluconic acid, cotton seed oil, sugarcaneand beet molasses) on PHB yield was shown in Fig. 4. Among the tested carbon sources, beet and sugarcane molasses were found to be the best carbon sources for PHB production with yield of 60%, 54.74%(w/w) respectively followed by glucose as a carbon source with PHB yield of (52.94% w/w). Beet molasses is an industrial waste and economic carbon source which leads to reduction of production cost. On the contrary, several workers found that simple sugars like glucose and glycerol are easily used by bacteria to enhance both growth and production of the polymer(46, 47). Carbon sources are important as they have three different functions within the microorganism which are: biomass synthesis, maintenance of the cell and PHA polymerization (7). One of the main problems for the extensive production PHBs is their high production cost. The selection of the suitable carbon substrate is considered as an important factor as it highly

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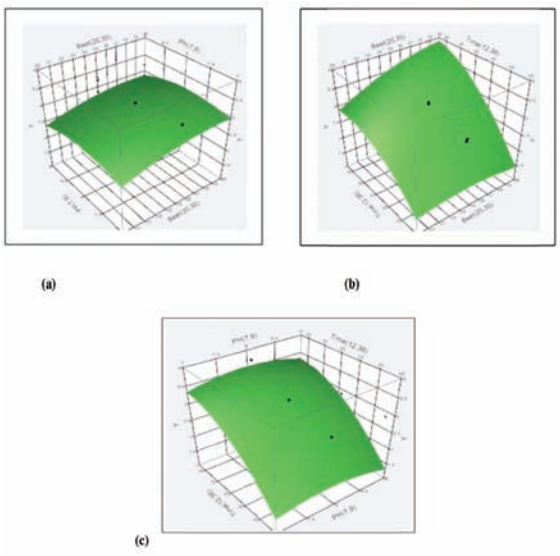

Fig. 9. (a-c): Three dimensional response surface curves revealing the effect of interactions of significant variables on production of PHB by *Bacillus aryabhattai* 6N-NRC (a) beet molasses concentration and pH (b) beet molasses concentration and time (c) pH and time.

affects the total cost of the produced PHB (48). Similar results were reported by Getachew and Woldesenbet (49)who obtained 56% PHB yield using pretreated sugarcane bagasse followed by corn cob with 52% yield. Yu *et al*. (50) showed that bagasse hydrolysate was used to produce 54% PHB yield.A strain of recombinant *E. coli* was reported to produce the polymer utilizingmolasses as source of carbon. The final dry cell weight, PHB content and productivity were 39.5 g/L, 80 wt% and 1 g/L/h, respectively (51).

Effect of different sources of nitrogen : To study the effect of nitrogen source for maximum PHB production, different organic and inorganic nitrogen sources were included in the MSM medium along with the best C-source (beet molasses), and the results are depicted in Fig. 5. Among all the tested nitrogen sources, NH_4Cl was found to be the best source of nitrogen which gave the highest yield of PHB (60%). This result agrees with Musa *et al*. (32) who reported that maximum PHB production was shown by NH_4Cl because it represents a readily utilizable nitrogen source using *Citrobacter*sp. and *Bacillus* sp.2. Species utilizing ammonium (NH_4^+) containing nitrogen source can be advantage with respect to industrial applications where in ammonia containing waste liquids can be utilized for the production of PHB (31). Mulchandani *et al.* (52) and Raje and Srivastava (53) by using *A. eutrophus* obtained highest PHB yield using ammoniumsulphate followed by ammonium chloride.

Effect of inoculum size: The inoculum size of the selected isolate was studied to stabilize intial microbial load 1% (v/v) was the best intial inoculum size where maximum PHB output was 73.96%. However, minimal PHB accumulation (52.27%) was achieved with 15 % (v/v) inoculum size (Fig.6). This may be attributed to the fact that a small size of inoculum may results in a number of microbial cells which is insufficient and therefore a low amount of secreted enzymes, On the contrary larger size of inoculum may cause oxygen reduction and nutrients depletion in the fermentation media (54).

Determination of fermentation factors affecting PHB production using Plackett-Burman design: To reduce the production cost and maximize the yield it is important to optimize the fermentation medium and conditions. The recent optimization studies have depended on statistical experimental design and response surface analysis. Statistical design is an efficient method to explain the major fermentation parameters and also their interactive effects on the process. It is an effective way to identify significant variables effective on PHB production, thus minimize the process development time and cost (55). Plackett-Burman design offers good and quick screening method and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component (56). The design is recommended

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when more than five factors are under investigation (57).

Screening of the most effective factors affecting PHB production was done using the Plackett-Burman design. Seven components were chosen for the study where each variable was tested at two levels, high concentration (+) and low concentration (-) in 8 trials and all the experiments were done in triplicate, and average of the results was used as the design response (Table 3). The variation in PHB production was ranging from 0.164 to 3.252 g/l. This showed that these parameters have a strong effect on production of PHB. The maximum production of PHB was observed in trial ordered 4 where the production of PHB and percentage yield of PHB increased to 3.252g/L and 79.07% respectively.

Analysis of the effect of the physicochemical parameters of the media showed that the production of PHB is highly affected by time, beet molasses concentration and pH. On the other hand, concentration of NH_4Cl, KH_2PO_4 and Na_2HPO_4 and temperature adjustment had relatively lower effect (Fig. 7). Many investigations showed that both pH and incubation time had significant influence on PHB production (58, 59, 60). However, other reports pointed critical importance of carbon source on PHB production (61, 62).

Depending on Plackett-Burman experiment results a pre-optimized medium composed of: KH_2PO_4 1.5g; Na_2HPO_4 3.525g; $MgSO_4.7H_2O$ 0.2g; $CaCl_2$ 0.02g; NH_4Cl 0.75g; ferric citrate 0.0015g; trace elements solution 1ml; distilled water 1000 ml inoculated with inoculum size 1% (v/v) of 48 h old culture incubated on rotary shaker was used in response surface methodology (RSM) for further conditions optimization.

Response surface methodology: The 3 variables (Beet molasses concentration , pH and time) identified by Placket-Burman design experiment having higher main effect on PHB production were further tested and optimized through Box-Behnken design methodology . This method allows the interaction of three independent variables at three different levels low (-1), medium (0), high (+1)) were listed with 13 trials (Table 4)

Box – Behnken design of RSM was used to estimate the influence of each individual factor and also their interaction effects on PHB production by the selected isolate. Response surface analysis showed that beet concentration 30 g/l, pH value 8 and an incubation time of 36 hours were the optimum production conditions. Other variables were kept in their (zero) initial values. To validate the exact optimum values of Beet molasses, pH and time and their interactions, statistical designs were used and the ranges between the optimum points were selected. Therefore, Box-Behnken design was focused on the interaction between these three factors while other factors were effectively insignificant for PHB production. In this connection, optimization of the effective factors on production of the polymer and their interactions were tested by Lakshman*et al*. (63) and Prabisha *et al*. (60), Box-Behnken design was applied to detect the optimum level of each factor. Design-Expert software was used to design Box-Behnken matrix and PHB yields of all trials are given in Table 5. Multiple regression analysis was used to analyze the experimental results and the second-order polynomial equation was formed to explicate the PHB yield through quadratic equation as follows:

$$Y = 3.0516222 + 0.2446023(X_1) - 0.043696(X_2) + 0.8283713(X_3) - 0.000305(X_1)(X_2) + 0.2515275(X_1)(X_3) + 0.0348075(X_2)(X_3) - 0.164019(X_1)^2 - 0.233841(X_2)^2 - 0.337969(X_3)^2$$

Where Y is the response (PHB yield), X_1, X_2 and X_3 are terms coding for the variables including beet molasses concentration, pH and incubation time respectively. The equation was found to be statistically significant by evaluation using F-test for analysis of variance, which shows that the regression analysisat 98% confidence level ($P<0.05$) is highly significant as shown in Table 5 and 6. ANOVA for yield of PHB showed that F value of 32.0101 with a low probability value and

P-value of the quadratic regression model (P>F 0.0079) which proves the strong significance of the model. The R^2 value of 0.989 (so near to1) which confirms the model accuracy showed a strong correlation between the actual and the predicted values of PHB yield. Therefore, the model fits 98.9% of the total variations in PHB yield. The optimum values of the various variables differ according to the microorganisms selected for production of PHB and their place of isolation and in turn alter their physiological characteristics (64). The Actual by Predicted plot provides a visual assessment of model fit that reflects variation due to random effects. Fig. 8 plots the actual values of response (PHB production) against its marginal predicted values.

To determine the optimum values of beet molasses concentration, pH and incubation time for optimum yield of PHB and studying their interactions, three dimensional response surface curves were plotted as illustrated in Fig. 9(a, b, c). Fig. 9(a) shows that the increase in pH and beet molasses concentration upto 8 and 30 g/L respectively increased the yield of PHB. On the

Table 1. Isolation and screening of PHB producing isolates

Soil sample	**Total No. of different types of isolates**	**No. of PHB positive isolates (producer)**	**No. of PHB negative isolates (non-producer)**
Soil A	7	3 (A1toA3)	4
Soil E	8	2(E1,E2)	6
Soil G	5	3(G1,G4,G5)	2
Soil J	4	1(J3)	3
Soil K	6	2(K2,K5)	4
Soil N	8	4(N1 to N3, N6)	4

Table 2. Biomass and PHB production by 15 positive bacterial isolates obtained from different soil samples

S.no.	Soil sample	PHB positive	PHB (g/L) isolate	Biomass (g/L)	PHB yield (%)
1	A	1	0.092	0.6815	13.5
2		2	0.109	0.6813	16
3		3	0.158	0.79	20
4	E	1	0.149	0.582	25.6
5		2	0.163	0.519	31.4
6	G	1	0.152	0.527	28.8
7		4	0.115	0.6534	17.6
8		5	0.126	0.689	18.3
9	J	3	0.098	0.624	15.7
10	K	2	0.135	0.572	23.6
11		5	0.086	0.672	12.8
12	N	1	0.160	0.575	27.8
13		2	0.122	0.589	20.7
14		3	0.148	0.655	22.6
15		6	0.17	0.44	38.63

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Table 3. Nine trial Plackett-Burman design matrix for seven variables predicted for PHB production *Bacillus aryabhattai*

Trial no	Beet molasses (g/L)	Ammonium chloride (NH_4Cl) (g/L)	Disodium hydrogen phosphate (Na_2HPO_4) (g/L)	Potassium dihydrogen phosphate (KH_2PO_4) (g/L)	pH	Time (days)	Temperature (°C)	Production (g/L)	Dry weight (g)	Yield (%)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7			
1	-(15)	- (0.5)	-(2)	+(2)	+(8.5)	+(3)	-(25)	0.26439533	2.302	11.485
2	+(25)	-(0.5)	-(2)	-(1)	-(6.5)	+(3)	-(25)	0.98512974	3.807	25.877
3	-(15)	+(1)	-(2)	-(1)	-(6.5)	-(1)	+(35)	1.68814559	3.182	53.053
4	+(25)	+(1)	-(2)	+(2)	+(8.5)	-(1)	- (25)	3.25168513	4.112	79.078
5	-(15)	-(0.5)	+(5)	+(2)	-(6.5)	-(1)	+(35)	1.7011752	3.301	51.535
6	+(25)	-(0.5)	+(5)	-(1)	+(8.5)	-(1)	+ (35)	2.28684118	3.012	75.924
7	-(15)	+(1)	+(5)	-(1)	-(6.5)	+(3)	- (25)	0.16370211	2.34	6.996
8	+(25)	+(1)	+(5)	+(2)	+(8.5)	+(3)	+(35)	1.21428571	3.816	31.821
9*	20	0.75	3.525	1.5	7.5	2	30	2.13	2.88	73.960

Table 4. Box-Behnken design of significant variables affecting production of PHB by Bacillus aryabhattai

Trial	Independent Variables			Yield (g/L)
	Beet (X1)	PH (X2)	Time(X3)	
1	(-) 20	(-) 7	(0) 24	2.396
2	(+) 30	(-) 7	(0) 24	2.919
3	(-) 20	(+) 9	(0) 24	2.868
4	(+) 30	(+) 9	(0) 24	2.888
5	(-) 20	(0) 8	(-) 12	1.826
6	(+) 30	(0) 8	(-) 12	1.779
7	(-) 20	(0) 8	(+) 36	2.840
8	(+) 30	(0) 8	(+) 36	3.799
9	(0) 25	(-) 7	(-) 12	1.701
10	(0) 25	(+) 9	(-) 12	1.439
11	(0) 25	(-) 7	(+) 36	3.427
12	(0) 25	(+) 9	(+) 36	3.306
13	(0) 25	(0) 8	(0) 24	3.005

contrary, the more increase or decrease in these two values lowered PHB production. Fig. 9(b) shows the interaction between beet molasses concentration and time, which depicts the highest beet concentration at incubation time of 36h whereas any variation of these values decreased the produced PHB. Fig. 9(c) shows the interactive effect of time of incubation and pH. Thus the best values of the tested variables were beet molasses concentration 30g/L, pH 8 and incubation time 36h to obtain 3.7992 g/L of PHB. Hassan *et al*. (64) reported the great influence of time of incubation on PHB production using *Bacillus subtilis*.Gouda*et al*. (65) found that both glucose and molasses used as sources of carbon have strong effects on biopolymer production. This may be attributed to the fact that PHB production is related to the high concentration of carbon source as well as molasses is considered a mixture of different salts as mineral source. This is comparable to maximum PHB production 5.41 g/L obtained by using molasses as source of carbon by *B. megaterium* in batch culture (66, 67).

Conclusion

A new bacterial strain which could produce PHB was isolated from Egyptian soil and was identified by using 16S rDNA technique as *Bacillus aryabhattai*. Response Surface Methodology approach through Box-Behnken design for optimization of synthesis of PHB showed high efficiency to increase PHB yield. The results indicated the impact of beet molasses concentration and the time of incubation on PHB production which is an economically and environmentally important product and can solve

Table 5. Analysis of variance

Source	DF (Degree of Freedom)	SS (Sum of Squares)	Mean Square	(R squared) R2	Adjusted R squared	F Ratio	Prob > F
Model	9	6.4884444	0.720938	0.989694	0.958776	32.0101	0.0079*

Table 6. Results for ANOVA analysis for optimization of PHB production by *Bacillus aryabhattai*

Term	Coefficient Estimate	Std Error	t ratio	P-value
Intercept	3.0516222	0.13934	21.90	0.0002*
X1 (Beet)	0.2446023	0.063472	3.85	0.0309*
X2 (pH)	-0.043696	0.058705	-0.74	0.5107
X3 (Time)	0.8283713	0.053059	15.61	0.0006*
X1X2	-0.000305	0.102394	-0.00	0.9978
X1X3	0.2515275	0.075037	3.35	0.0440*
X2X3	0.0348075	0.075037	0.46	0.6743
X12	-0.164019	0.10404	-1.58	0.2130
X22	-0.233841	0.105889	-2.21	0.1143
X32	-0.337969	0.094248	-3.59	0.0371*

one of the problems of the environment which is waste management. Using byproducts such as molasses reduces the production cost. Moreover, molecular approaches are recommended to improve microbial production of PHB.

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